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PRINCIPAL INVESTIGATOR: Piyush Gupta
Robert A. Weinberg, Ph.D.

CONTRACTING ORGANIZATION: Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

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6. AUTHOR(S)
Piyush Gupta
Robert A. Weinberg, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

E-Mail: gupta@wi.mit.edu

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13. ABSTRACT (Maximum 200 Words)
Breast cancer is a disease whose progression requires the involvement of many different cell types. These cell types, in addition to the mutated cancerous cells that initiate formation of the tumor mass, include non-cancerous blood vessel and connective tissue cells. These ancillary cell types, while not cancerous on their own, are required by the cancer cells in order for a tumor to grow beyond a very small size. Therefore, it is important to understand the interactions between these two cancerous and non-cancerous cellular components of a breast tumor mass, since such interactions may serve as novel targets for therapeutic intervention. The proposed work concerns the origins of the tumor-associated stroma.

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INTRODUCTION:

The progression of a breast tumor is dependent upon the participation of many different cell types. These cell types, in addition to the genetically mutated epithelial cells that initiate the tumor, include endothelial cells, fibroblasts, macrophages, and mast cells. A growing body of work has implicated these non-tumorigenic stromal cells as critical mediators of carcinoma progression(1, 2, 3, 4, 5). For example, endothelial cell mediated neo-vascularization of a growing tumor mass is critical for the tumor to receive the oxygen and other nutrients it needs to grow beyond a few millimeters in diameter. Mast cell secreted matrix metalloproteinases have been shown to be rate limiting in mediating the release of extra-cellular matrix bound pro-angiogenic factors(6, 7). Human prostate cancer associated fibroblasts have been shown to promote the tumorigenic progression of pre-neoplastic Simian Virus 40 Large T Antigen (SV40LTAg) immortalized prostate epithelial cells in a rat kidney capsule model of human prostate cancer(5, 7). These examples and others highlight the importance of understanding the bidirectional interactions between carcinomatous epithelial cells and normal host tissues in the process of breast tumorigenesis.

Characterization of the epithelial-stromal interactions described above requires, as a prerequisite, knowledge of the origin of the tumor stroma. Certain elements of the tumor stroma such as macrophages, mast cells, and other cells of immune origin have a well-established circulation-derived origin(8, 9, 10). In contrast, it has traditionally been assumed that the mesenchymal lineages within the tumor stroma, such as fibroblasts and endothelial cells, are derived from normal tissues adjacent to the growing carcinoma. In this model, the carcinomatous breast epithelial cells are envisioned to secrete locally diffusible chemotactic factors into their immediate environment that induce the subsequent in-migration of nearby mesenchymal cells into the tumor mass(1, 4, 6).

There is, however, an alternative model for how the carcinomatous breast epithelial cells might induce the recruitment of mesenchymal cells into the tumor mass. In this model, in addition to the more proximally derived cells of the tumor mesenchymal stroma, the malignant epithelial cells recruit mesenchymal precursor cells from the circulation of the host, into which they have been deposited by distant sites within the body, such as the bone marrow. Once recruited, these cells are also subverted by the carcinoma cells and are exploited in much the same way as the more proximally recruited stromal cells to provide the survival and mitogenic signals required by the carcinoma cells in order for the latter to proliferate, invade, and metastasize.

BODY:

I have previously reported demonstrating that MCF-7+Ras breast carcinoma cells recruit stromal cells from the circulation into the growing tumor mass. Furthermore, as described in a previous report and expanded upon here, MCF-7+Ras breast cancer cells perturb the bone marrow and peripheral blood compartments in the host, leading to an increase in endothelial precursor cell (EPC) levels, but not a detectable increase in hematopoietic stem cell (HSC) levels. This model, originally considered highly speculative, had become attractive because of a number of relatively recent studies that had demonstrated the ability of circulating hematopoietic stem cells to contribute to a variety of lineages in the adult animal *in vivo*. Through either bone marrow transplantation or direct blood-stream injection of labeled cells, these studies had shown that hematopoietic stem cells are capable of giving rise to neurons, cardiac muscle, muscle, hepatocytes, mesangial cells, and various types of epithelial cells(11, 12, 13, 14, 15). Additionally, complementary experiments had shown that cells of neuronal or muscular origin are capable of giving rise to the entire hematopoietic system(16, 17). Taken together, these experiments originally suggested that adult stem cells are much more plastic and primitive than originally imagined.

However, more recent work has cast a shadow over these intriguing phenomena and has suggested that many of the surprising finding of plasticity *in vivo* are due to very rare cell fusion events, not transdifferentiation, as originally proposed. This conflict, which has still to be definitively resolved, is of central importance to the current work.

Progress in the past year with respect to the proposed Statement of Work (SOW):

Task 1:

As discussed and justified in last year's progress report, the experiments with the lewis lung carcinoma (LLC) cells were replaced with experiments using the MCF-7+Ras human breast carcinoma cell line (discussed further below). This is because upon histological and flow-cytometric examination, the LLC line failed to yield the requisite stromalized tumors required for the proposed work.

Task 2: As described in last year's progress report, preliminary experiments with the lacZ marker indicated that staining conditions could not be standardized for all cell types present in a tumor population, and that the GFP was much more readily visualized in either fresh of fixed and frozen tissue sections. Therefore, Task 2 was marginally modified by substituting lacZ labels with GFP labels.

I have succeeded in creating immunocompromised chimeric C57Bl/6 mice in which the bone marrow of the mice is specifically labeled with the GFP marker (Months 1-18).

a. I Inter-crossed Rag-/- C57Bl/6 mice with GFP transgenic C57Bl/6 mice to produce Rag-/- GFP+ mice (Months 1-9). (See Figure 1 for an ochterlony assay for the evaluation of the Rag genotype in inter-crossed mice, as well as another dot-blot immunoassay.)

b. I created immunocompromised NOD/SCID mice with GFP labeled marrow by bone marrow transplantation using Rag-/- GFP+ C57Bl/6 mice as donor mice and NOD/SCID mice as recipient mice (Months 9-18). Please note that I have changed the strain of recipient mice from the C57Bl/6 Rag-/- in the original proposal SOW to the NOD/SCID strain. This is because NOD/SCID mice are traditionally better at accepting xenografts than Rag-/- and also, importantly, because we maintain a colony in-house that makes use of these mice cost-efficient. I have performed at least 100 transplants with these strains in the past year, and have been largely successful, as confirmed by GFP-positive cells in the peripheral circulation of the transplanted mice several weeks after irradiation.

Task 3: We have confirmed that the presence of a subcutaneous MCF-7+Ras tumor mass results in the mobilization of Sca-1+CD31+ endothelial precursor cells from the marrow into the circulation (Months 1-6). Furthermore, we have confirmed similar observations using the HMLER genetically defined mammary carcinoma cell line (Figure 2,3).

Task 4 Creation and immunohistochemical analysis of tumors created in the chimeric mice described in Tasks 1 & 2. (Months 6-26)

a. I have succeeded in injecting chimeric mice that have GFP-labeled marrow (created in Task 1) with MCF-7+Ras tumor cells at a subcutaneous site.

i. I have harvested the resulting subcutaneous tumor mass as well as the lungs, liver, kidneys, and spleen of the animals

ii. I processed the organs by fixation in 4% paraformaldehyde and subsequent equilibration in 18% sucrose followed by embedding in OCT to ultimately make frozen sections of the GFP-labeled tissue fragments.

iii. I am in the process of staining the frozen sections with endothelial and fibroblast specific antibodies, in order to identify the specific cell types present in the tissue mass.

b. I have injected 20 immunocompromised chimeric mice, created as described in Task 2, that have GFP-labeled marrow with HMLER breast tumor cells at a subcutaneous site in half the mice and orthotopically in the mammary fat pad in half the mice. The mice have been injected with the tumor cells, and at the endpoint of the experiment, I will:

i. Harvest the resulting subcutaneous tumor mass, and perform whole-mount GFP visualization on tumor fragments.

ii. Create frozen sections of the GFP-labeled tissue fragments

iii. Stain the frozen sections with endothelial and fibroblast specific antibodies

Task 5

This task is still a work in progress, and there is no data at this point to report on this specific task.

Figure 1A. Ochterlony assay with sheep anti-mouse antibody loaded in the center wells of rosettes, and serum protein from bred GFP+/? and Rag +/? mice loaded in the 6 outer wells of rosettes. The presence of a precipitin line indicates antibody in the serum, and therefore the lack of such a line indicates a Rag -/- strain. Mouse #2 and 9 below are GFP+/? Rag-/- mice and were used to establish a strain of Rag-/- GFP+ mice.

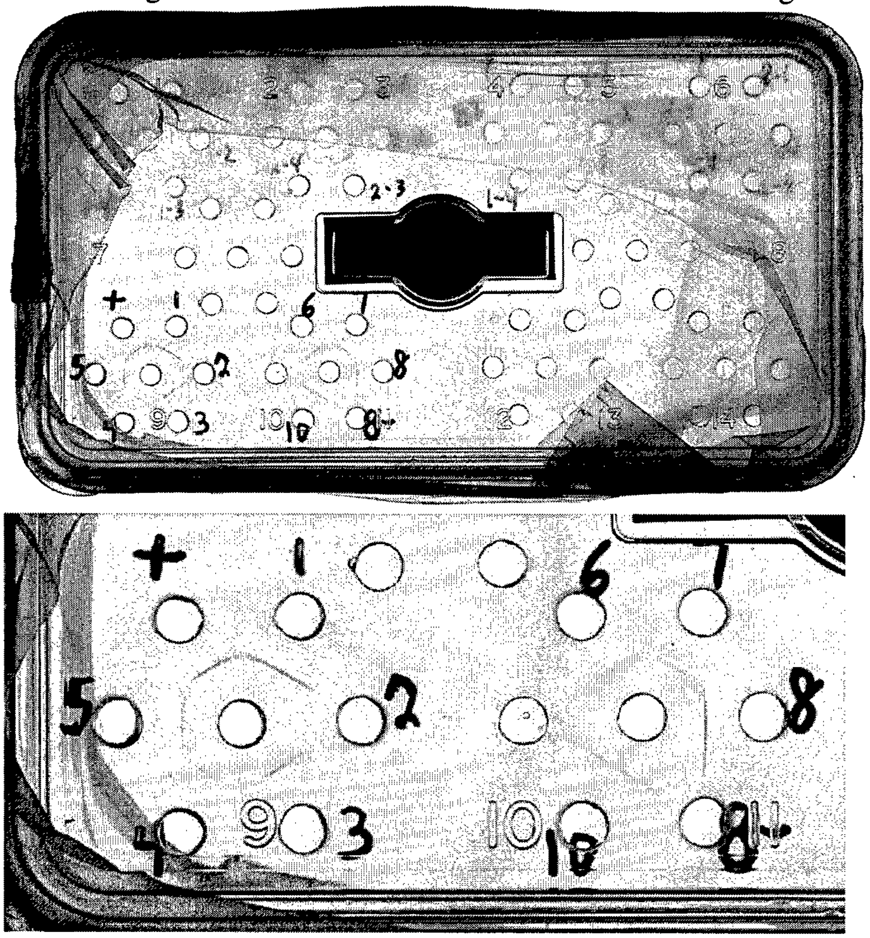
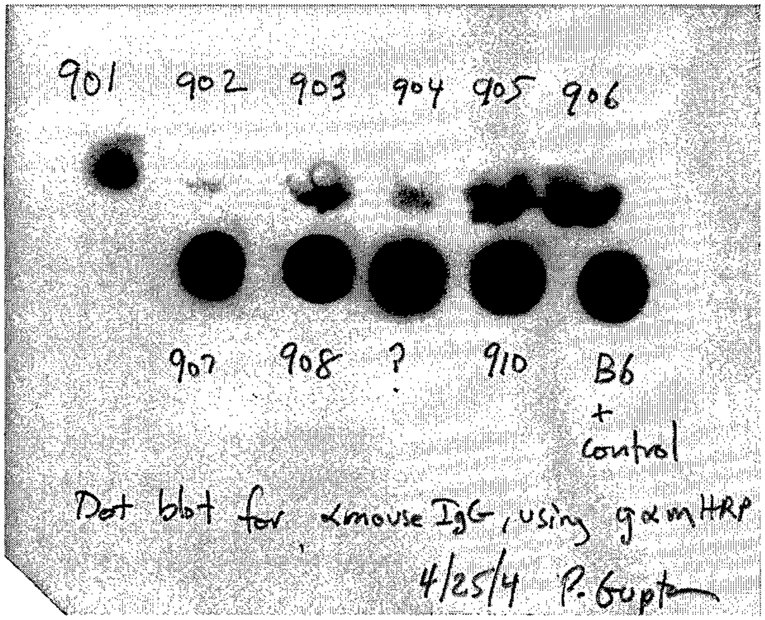


Figure 1B. Dot blot analysis of serum proteins in interbred mice, indicating the presence or absence of murine antibodies.



PERCENT OF POPULATION

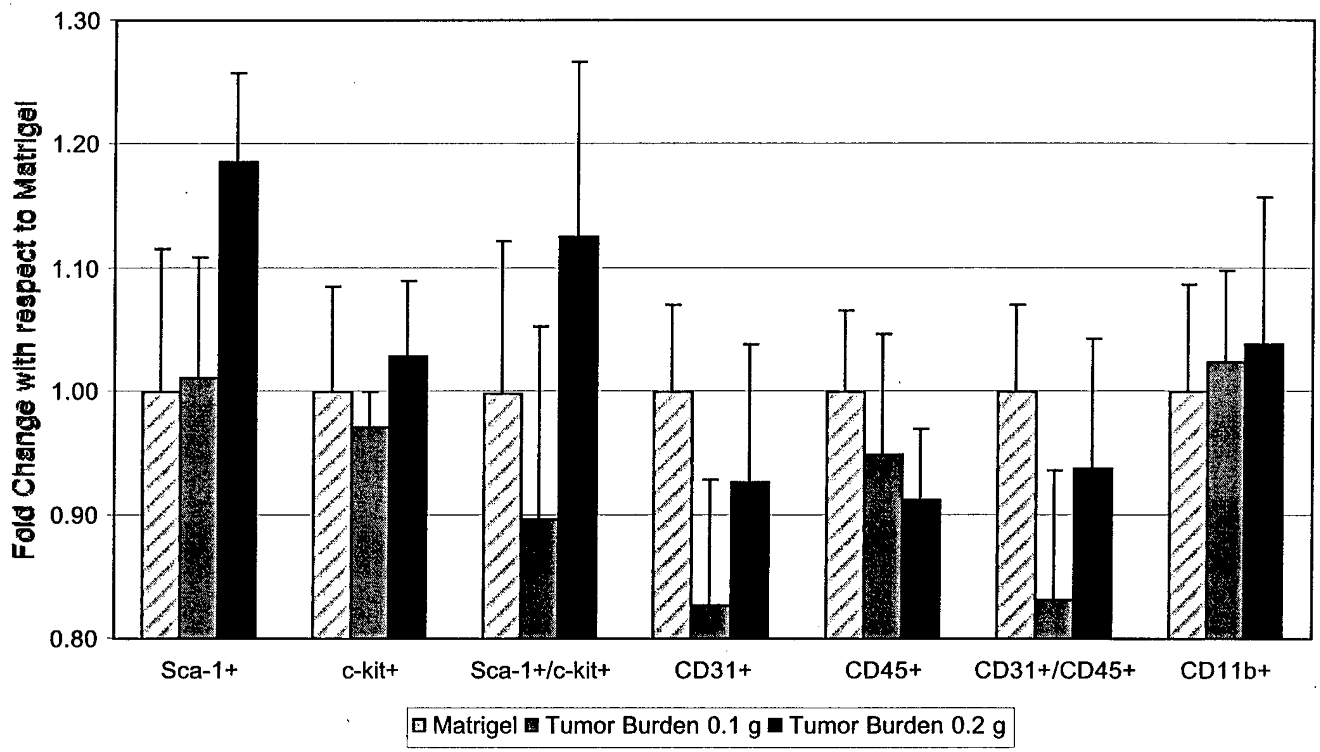
Sample	Sca+	CD31+	Sca+CD31+	Sca+CD31-	Sca+	Lin+	Sca+Lin+	Sca+Lin-
C6	6.9	10.64	3.63	3.27	0.67	39.28	0.62	0.05
C7	6.75	7.64	2.84	3.91	0.79	61.8	0.74	0.05
C8	5.81	8.68	3.1	2.71	0.75	33.99	0.61	0.14
C9	6.53	8.34	2.96	3.57	1.49	56.79	1.3	0.19
C10	5.97	7.05	2.51	3.46	1.16	57.35	1.02	0.14
C11	8.35	10.19	3.92	4.43	0.51	48.3	0.47	0.04
AVE	6.72	8.76	3.16	3.56	0.90	49.59	0.79	0.10
STDEV	0.91	1.41	0.52	0.58	0.36	11.07	0.31	0.06
CONF 95%	0.73	1.13	0.42	0.47	0.29	8.86	0.25	0.05

Sample	Sca+	CD31+	Sca+CD31+	Sca+CD31-	Sca+	Lin+	Sca+Lin+	Sca+Lin-
**3	5.49	8.09	3.22	4.5	1.12	51.25	1.06	0.06
**4	6.72	8.17	3.34	3.38	0.91	50.68	0.82	0.06
**5	8.55	10.06	4.59	3.96	1.46	46.51	1.31	0.09
**6	7.43	8.89	3.89	3.54	2.47	65.37	2.35	0.15
**7	12.76	10.18	5.49	7.27	3.76	64.97	3.42	0.34
**8					3.63	83.39	3.39	0.24
AVE	8.19	9.08	4.11	4.53	2.23	60.36	2.06	0.16
SEM	2.49	0.90	0.84	1.42	1.26	13.75	1.17	0.11
CONF 95%	2.18	0.79	0.74	1.25	1.01	11.00	0.93	0.09
p value	0.16	0.34	0.05	0.13	0.02	0.08	0.02	0.16

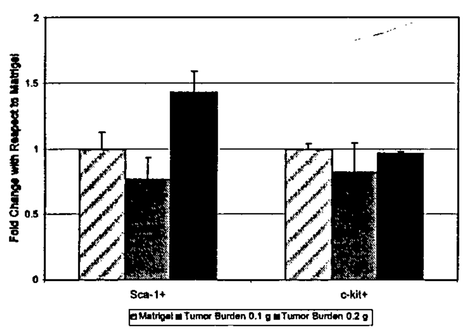
PERCENT OF TOTAL SCA+ POPULATION

	Sca+CD31+	Sca+CD31-	Sca+Lin+	Sca+Lin-
AVE	47.04	52.96	88.64	11.36
CONF 95%	6.23	6.94	27.68	5.64
AVE	50.13	55.31	92.51	7.04
CONF 95%	9.04	15.24	41.95	4.06

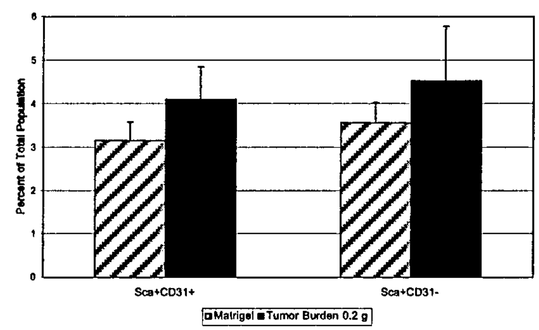
Relative Expression in Bone Marrow of Tumor-Bearing NOD-SCID Mice



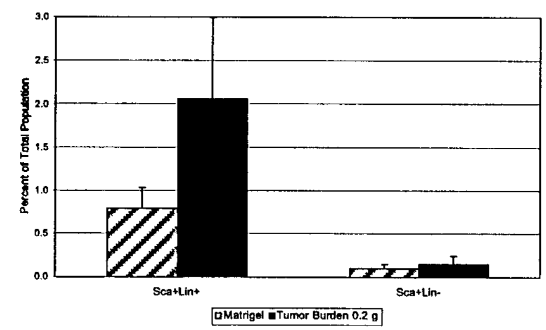
Sca+ in NOD-SCID Blood 041603



Distribution of Sca+ Bone Marrow Cells



Distribution of Sca+ Bone Marrow Cells



KEY RESEARCH ACCOMPLISHMENTS:

1. Demonstration that there is a perturbation of bone marrow and peripheral blood-derived cellular subfractions in the marrow and peripheral blood of breast tumor-bearing animals.
2. Demonstration that there is an increase in the levels of endothelial precursor cells in the bone marrow and circulation of breast tumor-bearing animals.
3. Demonstration that a significant fraction of the tumor stroma is comprised of cells that are derived from the circulation of a tumor-bearing animal.
4. Construction of GFP-positive Rag -/- immunocompromised strain for bone marrow transplantation procedures, for subsequent engraftment of human cancer cells.
5. Demonstration that there are increased levels of endothelial precursor cells in the stroma of tumors containing human carcinoma-associated breast fibroblasts, when compared to tumors containing normal human breast fibroblasts (with Akira Orimo).

REPORTABLE OUTCOMES:

Oral Presentations/Talks:
2002-3 Presentation to 5th year biology graduate students at MIT
Colrain mtg presentation, October, 2003

Poster Presentations:

1.Charlotte Kuperwasser, Tony Chavarria, Sandy McAllister, Piyush B. Gupta, Robert A. Weinberg. **Local and systemic effects during involution promote breast tumorigenesis**. (Poster). /Breast Research at Harvard - Transactions of the 1st Symposium/ April 9, 2004, Boston, MA.

2. 2003 Whitehead Institute Retreat
3. 2002 Whitehead Institute Retreat

Manuscripts:

Akira Orimo, Piyush B. Gupta, Dennis C. Sgroi, Andrea L. Richardson, Robert A. Weinberg. **Stromal carcinoma-associated fibroblasts enhance tumor growth and angiogenesis through recruitment of endothelial progenitor cells.** *Cell* (manuscript under review).

CONCLUSIONS:

Work funded by this proposal has demonstrated that there are increased levels of circulating endothelial precursor cells in the peripheral blood and bone marrow of immunocompromised mice bearing MCF-7+Ras human breast carcinoma tumors. The precise mechanism behind this mobilization is not yet known. Furthermore, the presence of human fibroblasts in the stroma of xenografted human breast carcinomas facilitates this mobilization. The work funded has also demonstrated that significant numbers of circulation-derived cells are present in the stroma of MCF-7+ras breast cancers, as gauged by immunofluorescence of fixed, frozed tissue sections, as well as by flow cytometric analysis of dissociated tumors. This observation, which utilizes human tissue xenografted into immunocompromised, bone marrow-transplanted mice, is of intrinsic interest, both from the scientific and therapeutic points of view, and addresses a key question in cancer pathobiology. Finally, we imagine that the GFP-labeled Rag-/- immunocompromised mouse strain created through this funded work may be of use to other labs that engraft human tissues into mice.

REFERENCES:

1. B. Elenbaas, R. A. Weinberg, *Exp Cell Res* **264**, 169-84. (2001).
2. H. C. Crawford, L. M. Matrisian, *Invasion Metastasis* **14**, 234-45. (1994).
3. K. J. Cullen, M. E. Lippman, *Cancer Treat Res* **61**, 413-31. (1992).
4. A. Noel, J. M. Foidart, *J Mammary Gland Biol Neoplasia* **3**, 215-25. (1998).
5. A. F. Olumi, et al., *Cancer Res* **59**, 5002-11. (1999).
6. L. M. Coussens, et al., *Genes Dev* **13**, 1382-97. (1999).
7. L. M. Coussens, C. L. Tinkle, D. Hanahan, Z. Werb, *Cell* **103**, 481-90. (2000).
8. M. W. Bennett, et al., *J Immunol* **160**, 5669-75. (1998).
9. M. Nesbit, H. Schaider, T. H. Miller, M. Herlyn, *J Immunol* **166**, 6483-90. (2001).
10. S. Radoja, M. Saio, A. B. Frey, *J Immunol* **166**, 6074-83. (2001).
11. D. S. Krause, et al., *Cell* **105**, 369-77. (2001).
12. E. Lagasse, et al., *Nat Med* **6**, 1229-34. (2000).
13. I. Lemischka, *Proc Natl Acad Sci U S A* **96**, 14193-5. (1999).
14. N. N. Malouf, et al., *Am J Pathol* **158**, 1929-35. (2001).
15. T. Asahara, et al., *Embo J* **18**, 3964-72. (1999).
16. E. Mezey, K. J. Chandross, *Eur J Pharmacol* **405**, 297-302. (2000).
17. K. A. Jackson, T. Mi, M. A. Goodell, *Proc Natl Acad Sci U S A* **96**, 14482-6. (1999).
18. S. H. Barsky, R. Gopalakrishna, *Cancer Res* **47**, 1663-7. (1987).
19. Z. M. Shao, M. Nguyen, S. H. Barsky, *Oncogene* **19**, 4337-45. (2000).
20. S. H. Barsky, W. R. Green, G. R. Grotendorst, L. A. Liotta, *Am J Pathol* **115**, 329-33. (1984).
21. C. Kalka, et al., *Ann Thorac Surg* **70**, 829-34. (2000).
22. S. Ugurel, G. Rappl, W. Tilgen, U. Reinhold, *J Clin Oncol* **19**, 577-83. (2001).
23. R. T. Poon, et al., *Ann Surg* **233**, 227-35. (2001).
24. G. Holzer, et al., *Med Pediatr Oncol* **36**, 601-4. (2001).
25. M. A. Goodell, K. Brose, G. Paradis, A. S. Conner, R. C. Mulligan, *J Exp Med* **183**, 1797-806. (1996).
26. M. A. Goodell, et al., *Nat Med* **3**, 1337-45. (1997).